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13. ABSTRACT *(Maximum 200 Words)*
We hypothesized that aberrant expression of Hox genes in mammary epithelial cells results in the inappropriate regulation of a wide variety of Hox target genes; this could lead to a change from a normal to a preneoplastic, or from a preneoplastic to a neoplastic phenotype. The purpose of this proposal is to test this hypothesis by manipulating the expression of Hox genes such as Hoxa1, Hoxa5, or Hoxa11 in cells from hyperplastic (preneoplastic) lesions of the breast by employing a tetracycline regulated vector system. The goals of this research are: (1) to understand the functions of homeobox genes in normal and neoplastically transformed mammary epithelial cells; (2) to ascertain whether or not the overexpression of homeobox genes results in a neoplastic phenotype; and (3) to assess whether these Hox gene overexpressing cells respond to exogenous RA. In the past year, we analyzed and compared both p53+/+ and p53-/- breast tumors for Hox gene expression. In addition, we examined the influences of pituitary hormones and the carcinogen, DMBA, on Hox A cluster gene expression. Further, we also established a tetracycline inducible Hoxa1 overexpressing cell line. This new data is significant in that it may be employed to develop novel methods for regulating homeobox gene expression as a therapy for human breast cancer.

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INTRODUCTION

Our hypothesis is built upon the observations that Hox genes are broadly-active transcriptional regulators in tissue and organ developmental processes. Some of these genes are specifically up- or down-regulated in neoplastic cells but not in preneoplastic cells. The neoplastic cells show massive deregulation of gene expression, and the neoplastic cells interact with their local environment much like cells in organogenesis. Our hypothesis is that aberrant expression of Hox genes, such as *Hoxa11*, in mammary epithelial cells results in major deregulation of gene expression and is a significant contributing factor to the neoplastic phenotype. The goal of this proposed research is to gain new insights into the functions of specific Hox genes with respect to the regulation of the growth of mammary epithelial cells using cell culture and animal model systems. The purpose of the research is to gain new knowledge about the intracellular molecular events which occur in the process of mammary carcinogenesis and to utilize this knowledge to develop new therapies for breast cancer. The scope of this research includes two laboratories with complementary expertise, and both cell culture and mouse models of breast carcinogenesis. This annual report is being written in month 36 of this project.

BODY

Task 1. To examine the expression of Hox genes in the murine mammary tumor model and the consequences of Hox gene overexpression on the phenotype of mammary epithelial cells in cell culture (months 1-20).
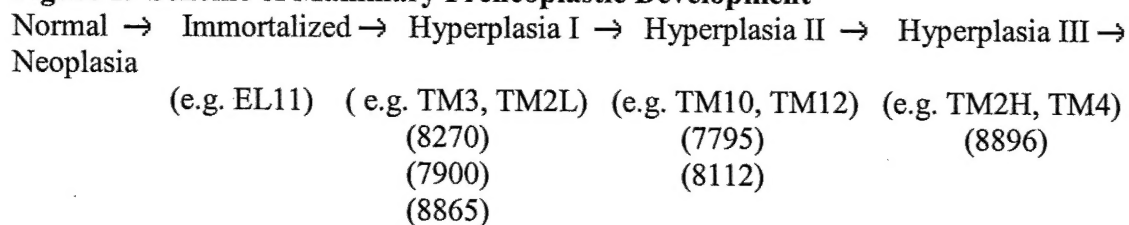
- Analysis of Hox gene expression in various murine tumors (months 1-24)
- Cloning of Hox genes into pUHD-10-3 (months 1-3)
- Generation and initial characterization of cell lines which stably express both pUHD-10-3/"Hox gene of interest" (i.e. Hoxa3 or Hoxa11, for example) and the tTA protein under tetracycline control (months 3-8)
- Assay of cell growth rate, cell morphology, cloning efficiency, and response to retinoic acid (months 9-16)

In Task 1, we have examined homeobox gene expression in mammary cell lines from a related animal model of mammary carcinogenesis, the p53 -/- mouse. Altered expression of the p53 tumor suppressor gene is often observed in human breast cancers, but mammary tumors were seldom observed in p53 -/- mice (1). Dr. Medina and Dr. Gudas's laboratories have analyzed Hox gene expression in cells derived from p53 -/- mammary epithelium transplanted into the cleared mammary fat pads of wild type (p53 +/+) BALB/c hosts. The mice were then left untreated, stimulated with hormones from pituitary isografts, or treated with the carcinogen DMBA (2). The cultured cells from this model expressed several different Hox mRNAs at higher levels than those from control, wild type mice. We do not have enough data from enough different cell lines to apply statistical tests at this time. However, we have repeated the Northern analyses on several cell lines and tumors three times to insure reproducibility. We are progressing with this research at the rate projected in the original proposal. There have been no major, significant problems in accomplishing this task.
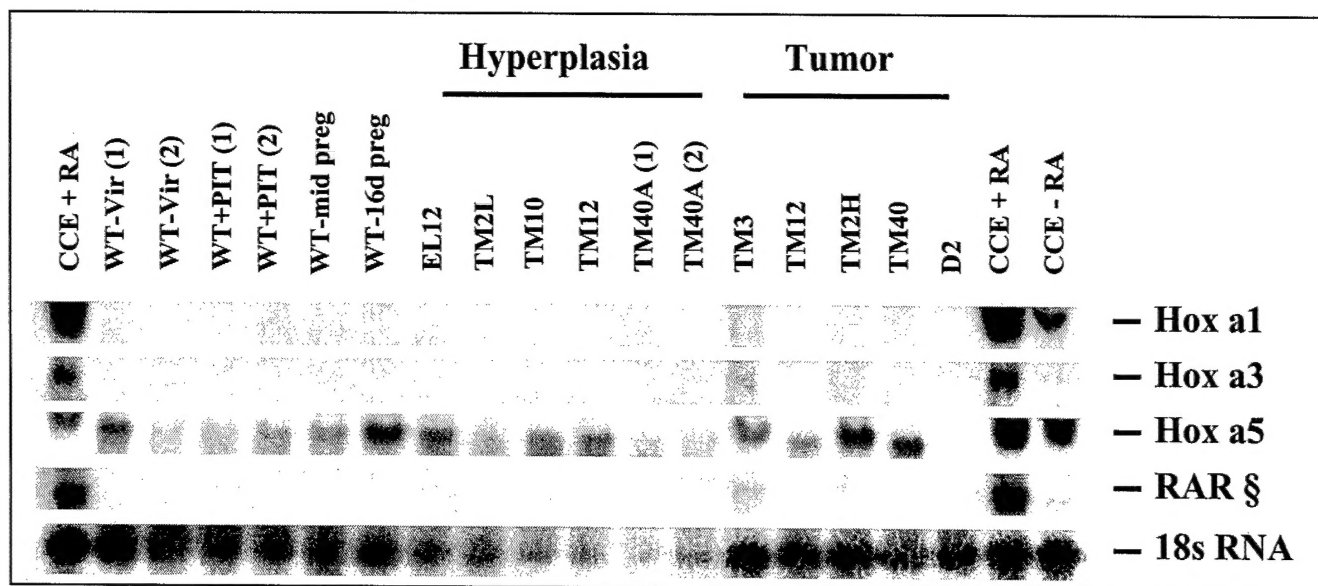
Recent experiments using the EL and TM series of transformed mouse mammary cells have demonstrated that the properties of immortality, hyperplasia and tumorigenicity are

independent and assortable characteristics. The scheme (Figure 1) for mouse mammary tumorigenesis has evolved to account for new information on preneoplasias. The scheme emphasizes the individual events which occur as preneoplasias evolve and suggests that preneoplasia is a heterogeneous and dynamic state. In this scheme, immortalized, nonhyperplastic cell populations, such as EL11, occur as an early event; hyperplasia I represents immortalized, hyperplastic, non- or very weakly- tumorigenic cell populations, such as TM3 and TM2L; hyperplasia II represents immortalized, hyperplastic cell populations with a weak to moderate tumorigenic potential such as D1, C4, TM10, TM12, and hyperplasia III represents immortalized, hyperplastic cell populations with a very high tumorigenic potential such as TM2H, TM4. It is not necessary that each preneoplasia or tumor proceed through all the steps sequentially.

Figure 1. Scheme of Mammary Preneoplastic Development

Normal $\rightarrow$ Immortalized $\rightarrow$ Hyperplasia I $\rightarrow$ Hyperplasia II $\rightarrow$ Hyperplasia III $\rightarrow$ Neoplasia

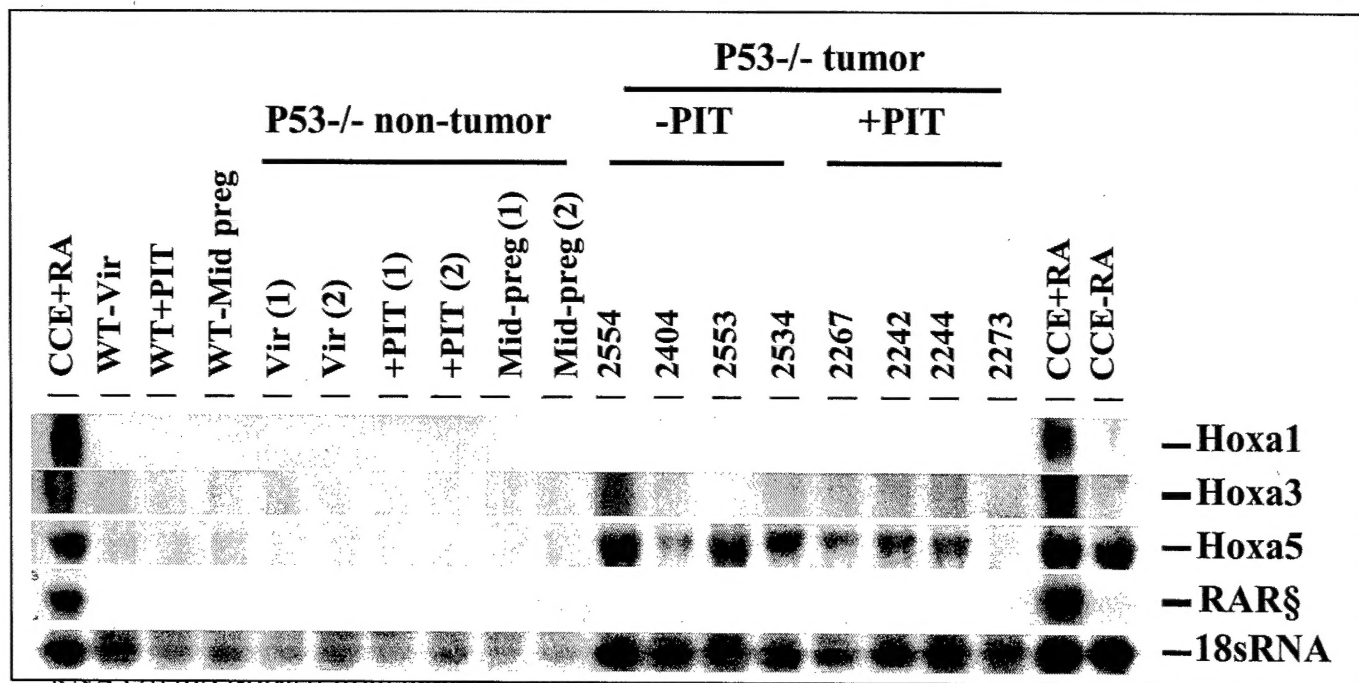|	(e.g. EL11)	(e.g. TM3, TM2L)	e.g. TM10, TM12)	(e.g. TM2H, TM4)
		(8270)	(7795)	(8896)
		(7900)	(8112)	
		(8865)		

Our data to date indicate that Hoxa5 mRNA is expressed in various types of breast tumors in wild type mice. Cells with low tumor potential, such as TM3 (7900), intermediate tumor potential, such as TM10 (7795), and high tumorigenic potential, TM2H (8896), all express high levels of the Hoxa5 mRNA (Figures 2, 3). Hoxa1 mRNA is expressed in a subset of these cells, including 7900 and 6787. Hoxa5 mRNA is expressed in normal tissue from wild type mice, especially during pregnancy. These data suggest a role for Hoxa5 in normal breast function. Intriguingly, no Hoxa1 or Hoxa3 mRNA expression was observed in the control wild type mice, either in virgin mice, pregnant control mice, or in mice in which involution of the breast is occurring (Figure 2).

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	CCE + RA	WT-Vir (1)	WT-Vir (2)	WT+PIT (1)	WT+PIT (2)	WT-mid preg	WT-16d preg	EL12	TM2L	TM10	TM12	TM40A (1)	TM40A (2)	TM3	TM12	TM2H	TM40	D2	CCE + RA	CCE - RA	
									Hyperplasia					Tumor							

mouse normal breast tissue, breast tissue at different stages of hyperplasia, and breast tumors. CCE is a mouse embryonic stem cell line which served as an experimental control. Mid preg: 13-14 days of pregnancy, 16d preg: 16th day of pregnancy, Vir: virgin, PIT: pituitary isograft, WT: wild type, RA: retinoic acid. 1 μM of RA was used to treat the CCE cells. 18S RNA was used as an internal standard for loading normalization.

When we examined the tumors from p53 -/- mammary cells implanted into the fat pads of wild type mice, we observed that Hoxa3 and Hoxa5 mRNAs are expressed in these p53 -/- tumors (Figure 3). This was true whether the mice were treated with pituitary extract or not, though the Hoxa5 levels were higher in p53 -/- breast tumors from animals without pituitary extract. Hoxa11 mRNA is expressed in many tumors as well, in p53 -/- cells implanted in WT fat pads. We were not able to correlate the expression of these homeobox genes with the severity of the tumor phenotype. We also were not able to correlate the expression of the Hoxa1, a3, a5, a9, and a11 genes with the presence or absence of a wild type, functioning p53 gene. However, we did determine that Hoxa5, unlike the other Hox genes, is expressed in some stages of normal breast development. Hoxa5 mRNA is overexpressed in the p53 -/- breast tumors to a greater extent than in the wild type breast tumors

P53-/- tumor

	P53-/- non-tumor										-PIT				+PIT					
	CCE+RA	WT-Vir	WT+PIT	WT-Mid preg	Vir (1)	Vir (2)	+PIT (1)	+PIT (2)	Mid-preg (1)	Mid-preg (2)	2554	2404	2553	2534	2267	2242	2244	2273	CCE+RA	CCE-RA
—Hoxa1																				
—Hoxa3																				
—Hoxa5																				
—RAR§																				
—18sRNA																				

RNA (20 μg) isolated from mouse normal breast tissue, p53 mutant breast tissue, and p53 mutant tumors. 1 μM of RA was used to treat the CCE cells. PIT: pituitary isograft. The Hox gene expression is normalized for loading to 18S mRNA.

Since a chemical carcinogen DMBA (7,12-dimethylbenz(α)anthracene) can increase tumor incidence (2), we asked whether or not the carcinogen, DMBA, influenced the expression of these Hox A-cluster genes. The wild type mice that had had their fat pads implanted with tumors from p53 -/- mammary cells were treated with DMBA. We found that Hoxa5 mRNAs were expressed in these p53 -/- tumors (Figure 4). However, we didn't find any significant differences when we compared the Hoxa5 expression in p53-/- tumors from the DMBA treatment, compared to its expression without drug treatment, or to its expression with pituitary isograft treatment (Figure 4).
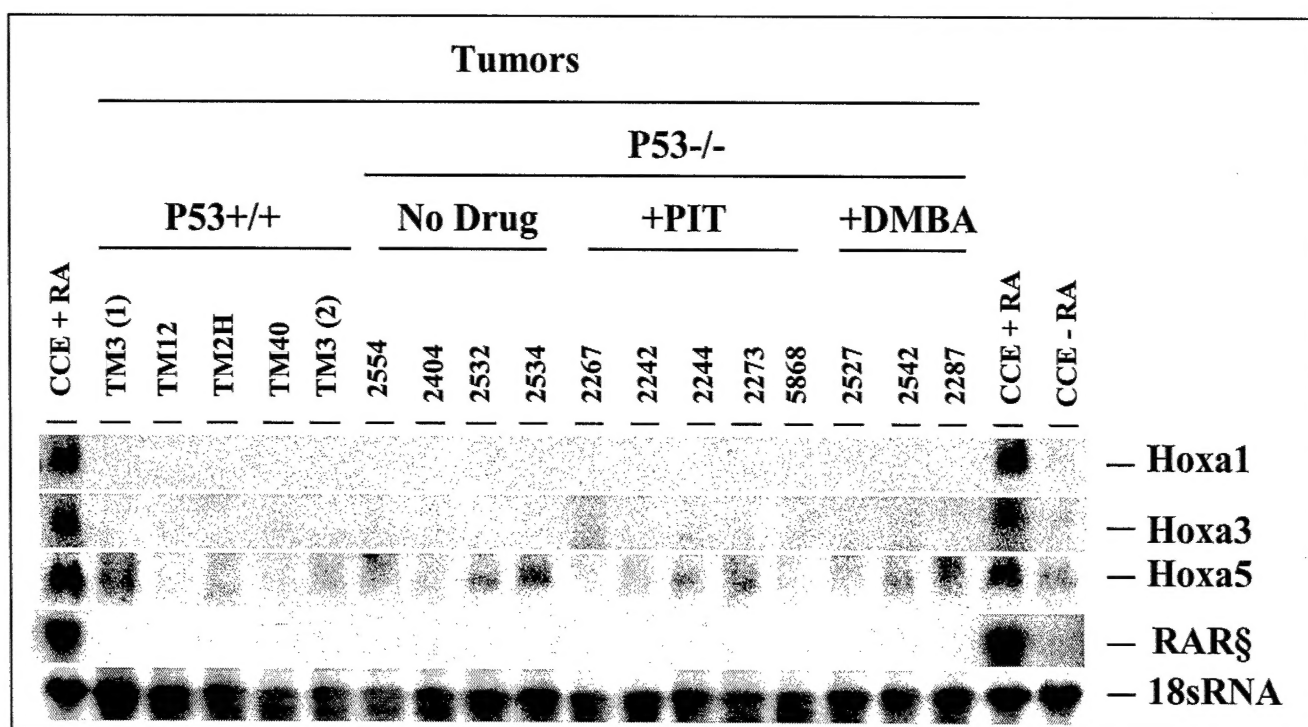
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Figure 4. Comparison of Hox A cluster genes and retinoic acid receptor β gene expression in mouse breast tissues or tumors of p53 wild type and mutant mice under the various conditions. Northern blot analysis of total RNA (20 μg) isolated from p53+/+ breast tumors, or from p53 mutant (p53-/-) breast tumors either with drug treatment or without drug treament. 1 μM of RA was used to treat the CCE cells. PIT: pituitary isograft. DMBA: 7,12-dimethylbenz-(α)anthracene. The Hox gene expression is normalized for loading to 18S mRNA.

Data from figure 1 to figure 3 were quantified by using densitometry with Image 1.62 software from the National Institutes of Health (NIH), and ready in publication format.

In Task 1, we are also artificially manipulating the expression of specific Hox genes in cells from hyperplastic (pre-neoplastically transformed) lesions by employing the tetracycline-inducible vector system (3-5). The TM10 preneoplastic outgrowth from which this cell line was derived has modest tumorigenic potential and is classified as a stage II hyperplasia. Full-length murine cDNA clones for the Hoxa1, Hoxa5, and Hoxa11 genes are available in this laboratory and are inserted into the plasmid pUHD-10-3, which contains a heptamerized tet operator upstream of a CMV minimal promoter; this is followed by a multiple cloning site and an SV40 polyadenylation sequence 3' of the multiple cloning site. Our first priority is to analyze Hoxa1, Hoxa5, and Hoxa11 (6). We have successfully made Hoxa1 overexpression lines in F9, a teratocarcinoma cell line

F9 cells were stably transfected by electroporation with this construct in addition to the tetracycline expression vector PTA-N, which contains the tetracycline/VP16 transactivator gene under the control of a tet heptamer sequence (3-5). This allows both the Hox protein of interest and the tet-VP16 transactivator protein to be regulated by the drug tetracycline. In the presence of tetracycline, no transactivator message or Hox cDNA should be expressed, but when tetracycline is removed from the medium the Hox gene expression is greatly induced (Figure 5).

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F9 stable cell lines which were shown by Northern blot to exhibit inducible expression of the Hox gene were then analyzed in culture with respect to their cell morphology, growth rate ([3H]thymidine uptake, cell number) over a 7-day period, and colony formation efficiency (14-day assay) (7). Cells stably transfected with only the PTA-N vector were used as a control.
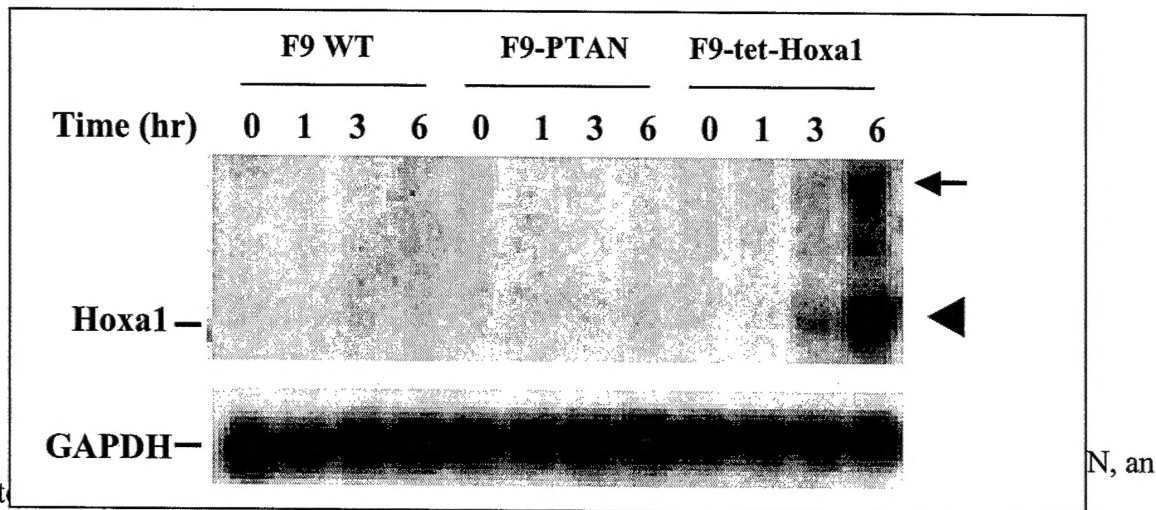
hybridized to random prime-labeled cDNA probe for Hoxa1. Arrow-head indicates the exogenous Hoxa1 mRNA, and arrow indicates the endogenous Hoxa1 mRNA.

We accomplished all the experiments proposed in Aim 1 except that we did not have time left to establish the Tet-inducible system in T10 cell line before the ending of this grant period. This was in part due to some problems of maintaining both the PTA-N vector and the Hox vector pUHD in the cells. In Aim 1, we extended some of our originally proposed experiments, such as the hormonal and carcinogen studies.

Task 2. To examine the consequences of Hox gene expression on the growth of epithelial cells in an animal model; tumor incidence will be assessed (months 9-36).

As described above, for Hoxa1, Hoxa3, and Hoxa5, we do not find a correlation with the tumor potential (high tumorigenicity) and the level of specific Hox A cluster gene expression (Figure 2).

Task 3. To examine gene expression in the Hox overexpressing cells. The tumor samples will be assayed directly from the animal, and cell lines will be made from the mammary carcinomas which overexpress different Hox genes. Gene expression studies aimed at measuring markers such as gelsolin, PKCδ, p96, C/EBPβ3, RARα, RARβ, RARγ, and cyclin D1 will be examined (months 25-36).

We initiated this task with the cells that express different tumor potential and different levels of expression of Hox genes, both the wild type and p53 -/- mammary cells. We have examined RARβ and RARγ mRNA levels to date (see Figure 2, 3, 4 for RARβ).

KEY RESEARCH ACCOMPLISHMENTS

(a) Measurement and detection of homeobox (Hox) gene expression, including Hoxa1, Hoxa3, and Hoxa5, at the mRNA level in various murine mammary cell lines at various stages in the carcinogenesis process in wild type (WT) mice.

(b) Detection of specific Hox A cluster gene expression at the mRNA level in cultured mammary cell lines from p53 -/- mammary epithelium in the mammary fat pads from WT mice.

(c) Determined the influences of hormone from pituitary, and the chemical carcinogen, DMBA, on Hox-A cluster gene expression.

(d) Generating Hoxa1 tet-inducible Hoxa1overexpression cell line.

(e) Testing of Hox gene expression in various cells with different tumor potential (Figure 1) by Northern analysis and by RT-PCR.

REPORTABLE OUTCOMES

Chen, S.W., Medina, D., and Gudas, L.J (2002). Homeobox Gene Expression in Mammary Tumor Cells from Wild Type and p53 -/- Mice at Various Stages of Carcinogenesis. Era of Hope. Department of Defense Breast Cancer Research Program Meeting 2002, Orlando, FL. Paper is in preparation - will be submitted January 2003.

CONCLUSIONS

First, we have shown that there is Hoxa5 mRNA expression in normal breast development, and that the Hoxa5 mRNA level is higher during pregnancy. Second, we have demonstrated, in two different mammary tumorigenesis animal models, that other homeobox gene expression (e.g. Hoxa1, a3, a5, a9, a11), including Hoxa5, is aberrantly high relative to expression in normal, control, non-tumorigenic and/or non-hyperplastic mammary cells. These data are important for understanding why breast epithelial cells sometimes become deranged and form tumors.

In the p53 -/- breast cancer model, we also have found effects of hormones on the expression of Hox genes. The addition of pituitary extract suppressed Hoxa5 mRNA expression relative to mice not treated with pituitary extract. Another laboratory has also reported related data (8), though they showed that Hoxa5 was required for p53 expression.

In addition to the gaining of fundamental knowledge about the process of carcinogenesis, these Hox A cluster genes could be biomarkers of different stages of the carcinogenesis process and/or targets for future pharmacological or gene therapy for breast cancer treatment. For homeobox genes of the B and C clusters, data similar to ours have been generated, i.e. re-expression of Hox B3, B4, and C6 genes was observed in human breast carcinomas (9-12). Additionally, it was recently shown that the Hoxa5 protein regulates the expression of the progesterone receptor gene at the transcriptional level (13), providing a potential selective growth advantage for the re-expression of high levels of Hoxa5 mRNA in the mammary tumors, which we observed. Higher progesterone receptor levels should lead to greater tumor growth in response to endogenous progesterone levels and tumors expressing Hoxa5 should have higher progesterone receptors.

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